

BIOCATALYST AND ITS USE IN ENZYMATIC RESOLUTION OF RACEMIC BETA-LACTAMS

Field of the Invention

This invention relates to a process for the production of optically active β -lactams by enzymatic resolution of the racemic β -lactam.

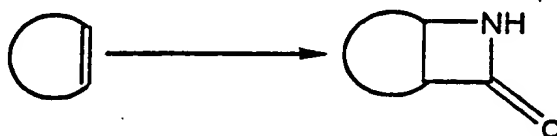
5 **Background of the Invention**

The utility of the β -lactam ring as a structural feature in biologically important compounds is well established, as is the synthetic utility of β -lactams. For example, the utility of β -lactams as masked β -amino acids is illustrated by their use as precursors to the C-13 taxol side chain.

10 The active molecules are often chiral, and it is preferable that they are accessible in the form of single enantiomers. This feature is also utilised in the design of stereodefined scaffolds, to generate single enantiomer compounds libraries for initial lead identification as part of a drug discovery programme.

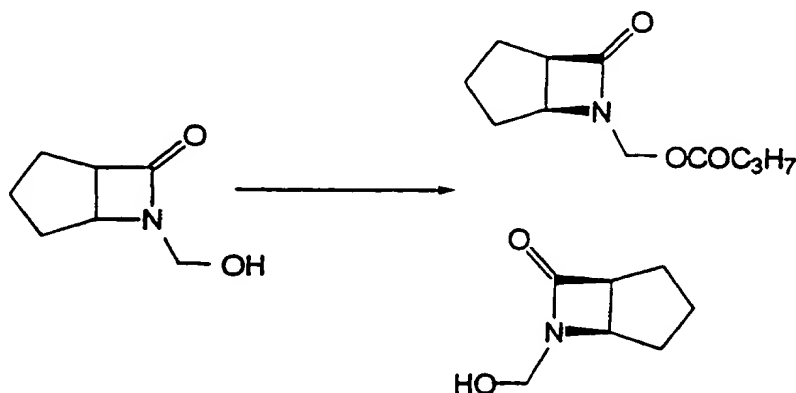
15 Alicyclic β -amino acids can be cyclised to the corresponding β -lactams, typically by use of carbodiimide reagents. They are also compounds having biological or pharmaceutical activity. For example, the natural product cispentacin, (-)-(1*R*,2*S*)-2-aminocyclopentanecarboxylic acid, has potent antifungal properties. It is synthesised as a natural product in *Streptomyces setonii* and *Bacillus cereus*, and is inhibitory to strains of *Candida*. The same amino acid has also been used to probe the relationship between
20 structure and taste in L-aspartyl dipeptides, where the absolute structure of the β -amino acid strongly affected the taste of the dipeptide.

Optically pure alicyclic β -amino acids may be made, for example, by the enzyme-catalysed solvent-based bioresolution of the amino carboxyl esters (Kanerva *et al.*, *Tetrahedron: Asymmetry*, 1996, 7, 1705). Such racemic amino esters are readily
25 synthesised from β -lactams of formula (1), which are themselves synthesised by cycloaddition of the appropriate cyclic alkene and chlorosulfonyl isocyanate, followed by reductive work-up using sodium sulfite:

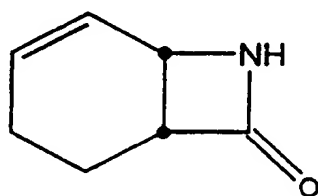


(1)

It is generally preferable to resolve enantiomers as early as possible in a synthetic sequence, to avoid having to perform the chemistry on the racemate at twice the scale as for the enantiomer. Thus, it is useful to be able to access β -lactam precursors of β -amino acids in resolved form. One approach is to derivatise the β -lactam to the *N*-hydroxymethyl- β -lactam using paraformaldehyde (Csomós *et al.*, *Tetrahedron: Asymmetry*, 1996, 7, 1789). The resulting primary alcohols can then be resolved using a lipase, for example to access the *O*-acylated precursor to cispentacin, as depicted below. Besides the extra derivatisation and deprotection required, the resolution with lipase AK requires high enzyme loading and chromatographic separation of the products, making this general approach unattractive at scale.



WO-A-97/10713 and EP-A-0232017 disclose various bicyclic compounds, including 7-azabicyclo[4.2.0]oct-4-en-8-one of the formula

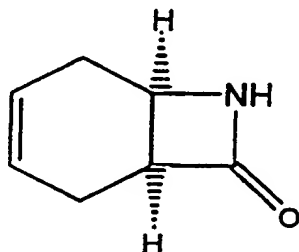


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These compounds are in racemic form. WO-A-97/10713 includes reference to enantiomers, but there is no evident means of resolution. For example, the compounds will not readily form salts with chiral resolving agents.

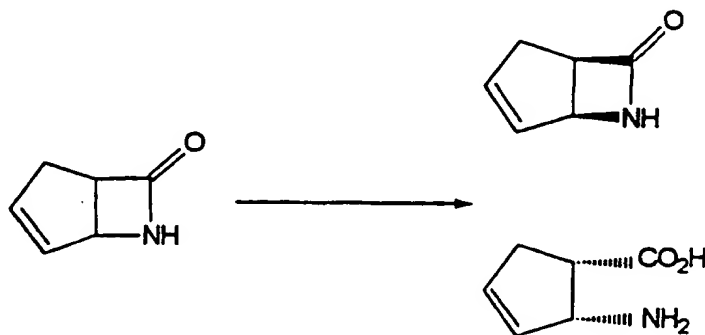
EP-A-0002564 discloses β -lactams, including bicyclic structures. It is suggested that the racemic lactams might be resolved, and refers to GB-A-1273278, but that depends on melt crystallisation, and probably requires that the substrate exists as a conglomerate.

Kurihara *et al*, Tetrahedron Lett., 1985, 26 (47), 5831, and also Tamara *et al*, Tetrahedron Lett. 1986, 27 (32), 3749, disclose a β -lactam, in single enantiomer form, of the formula



In the former paper, enzymatic desymmetrisation of a precursor (before conversion to a β -amino acid and then formation of the lactam ring) is involved. The latter involves classical resolution, using cinchonidine, of a ring-open precursor.

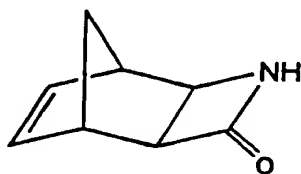
A more direct approach would be to resolve the lactam itself, thereby removing the separation problem evident in the work of Csomós *et al* (reference cited above), since the resulting amino acid is soluble in buffer, whilst the unreacted substrate can be extracted into solvent. This has been shown in one instance (Evans *et al*, *J. Chem. Soc., Perkin Trans. 1*, 1991, 2276; see also WO-A-92/18477) where *Rhodococcus equi* was used to provide (1*R*, 5*S*)-6-azabicyclo[3.2.0]hept-3-ene-7-one:



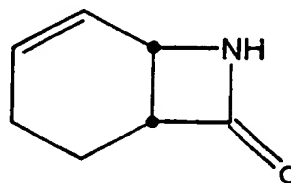
However, this biotransformation is an exceedingly slow process and is therefore not viable for operation on a commercial scale; 980mg whole-cell paste was needed to resolve 340mg racemate in 212 hrs, in order to recover unreacted lactam of >99% ee.

Summary of the Invention

The present invention is based on the discovery of a novel lactamase biocatalyst that allows efficient access to a range of synthetically useful β -lactams and the corresponding β -amino acids. A preferred embodiment of the invention is the application of this methodology to the preparation of novel single enantiomer cyclohexene-fused β -lactams. More particularly, the novel biocatalyst is capable of enantioselective hydrolysis of racemic lactams of the formulae (2) and (3)



(2)



(3)

A further aspect of the invention lies in the novel, enantiomerically enriched compounds of formulae (2) and (3), preferably as the levorotatory enantiomer.

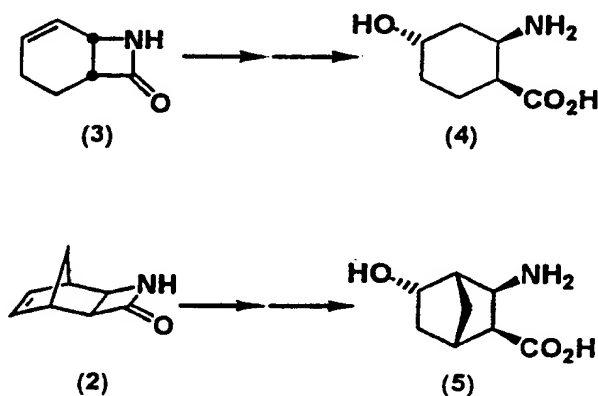
Description of the Invention

For identification of a suitable biocatalyst, a microbial screen was targeted at hydrolysis of racemic lactams (2) and (3). Hitherto, neither compound has been prepared in enantiomerically enriched form. Lactams (2) and (3) are conveniently synthesised by cycloaddition of chlorosulfonyl isocyanate with norbornadiene and cyclohexa-1,3-diene respectively (Stájer *et al.*, *Tetrahedron*, 1984, 40, 2385; Malpass *et al.*, *J. Chem. Soc., Perkin Trans. 1*, 1991, 2276).

400 Microbial strains were screened for hydrolysis of the lactams (2) and (3). Surprisingly, one strain of *Pseudomonas putida* not only hydrolysed the lactam, but did so selectively, with much higher activity than observed for the prior art strain of *Rhodococcus* (Evans *et al.*, reference as above). Thus, for lactam (2), when stirred with an equal weight of cell paste in phosphate buffer at 25°C, resolution was complete after 15 hours (>98% ee residual substrate), and the enantiomerically pure lactam extracted into

ethyl acetate in 38% yield. Similarly for lactam (3), after incubation with an equal weight of cells for 21 hours in buffer, the ee of residual lactam was >90%. Recovery of the enantiomerically enriched β -amino acids from aqueous solution is facilitated by conversion to *N*-Boc derivatives under standard conditions, followed by solvent extraction.

Enantiomerically enriched products obtained from the bioresolution of lactams (2) and (3) can be used to prepare information-rich chiral scaffolds for elaboration into single enantiomer compounds libraries. The alkene functionality is amenable to a range of transformations, especially through reaction with oxidants. For example, conversion of (3) to the chiral scaffold (4) gives three points for structural elaboration into defined regions of 3-dimensional space. Similarly, the more rigid scaffold (5) can be prepared via bioresolution of (2).



In summary, the present invention embodies a novel β -lactamase biocatalyst from *Pseudomonas putida*, combining the key attributes of high enantioselectivity with superior catalytic activity to β -lactamases described previously. This biocatalyst has been deposited (see Example 1). Use of this biocatalyst allows certain single enantiomer β -lactams to be prepared for the first time in synthetically useful amounts.

As will be evident to those skilled in the art, the biocatalyst can be used under conditions that can readily be determined, to produce a mixture of compounds. These can be separated by known methods. More particularly, the enantiomerically enriched β -amino acid produced by hydrolysis can be isolated. The isolated β -amino acid may then be subjected to a condensation reaction, to reform the β -lactam ring.

The biocatalyst can be used in whole cell or paste form. The enzyme can also be isolated, by techniques known to those skilled in the art.

The following Examples 1, 2, 4, 6 and 8 illustrate the invention. Examples 3, 5 and 7 illustrate the preparation of racemic substrates. TFAA is trifluoroacetic anhydride.

5 **Example 1** Biocatalyst

Glycerol stocks of 96 bacterial strains (obtained from the Applicant's strain collection) were used to inoculate 1.0ml Tryptone soya broth (Oxoid CM129) per well in 2.2ml 96-well plates (Advanced Biotechnologies AB-0661). These were then shaken at 25°C on a Heidolph Titramax 1000 incubator at max rpm for 45 hours. The cells were
10 harvested by centrifugation at 1000g, 4°C, for 10 minutes, and the cell pastes were stored at -20°C.

Cell pastes of the 96-well culture plates SCL0003 were resuspended in 0.5ml of 20g.L⁻¹ of substrate in 0.1M KH₂PO₄, pH 7.0 (13.6g KH₂PO₄ in 1.0L water, adjusted to pH 7.0 with 12M NaOH) and then shaken at 25°C on a Heidolph Titramax 1000 incubator
15 at max rpm for 40 (on the norbornadiene lactam (2) derivative) to 66 hours (on the cyclohexa-1,3-diene lactam (3) derivative). Reactions were stopped by diluting 1 in 10 in a 1:1 mix of MeOH:10mM KH₂PO₄, pH 7.0 (1.36g KH₂PO₄ in 1.0L water, adjusted to pH 7.0 with 12M NaOH). These were then assayed by HPLC. A 15cm 5µ Hichrom KR100 C8 Column was used with a running buffer of a 1:1 mix of MeOH:10mM KH₂PO₄, pH 7.0
20 (1.36g KH₂PO₄ in 1.0L water, adjusted to pH 7.0 with 12M NaOH) at a flow rate of 1.0ml.min⁻¹. Detection was at 210 nm. Results showed that a strain of *Pseudomonas putida* had reached 45% conversion on the norbornadiene lactam derivative and 43% conversion on the cyclohexa-1-3-diene lactam derivative. This strain, designated CMC 103381, was deposited at NCIMB Ltd., 23 St. Machar Drive, Aberdeen AB24 3RY, Scotland, on 31.03.99 and again on 29.03.00. The respective accession numbers are
25 41013 and 41040.

Example 2 Cell Paste

A 10µl loopful of colony of CMC 103381 (from agar plate) was used to inoculate seed flasks (100ml Tryptone soya broth (Oxoid CM129) per 500ml Erlenmeyer flask).
30 These were shaken at 25°C, 300rpm in a temperature-controlled shaker (New Brunswick G-25 with 25 mm (1 inch) throw) for 23 hours. A 0.67% v/v inoculum was used to inoculate the fermenters. The fermenters used were 3.0l Applikon fermenters containing

1.5L medium per fermenter. The fermentation medium contained, per litre: 15g yeast extract (Oxoid L21), 8g KH_2PO_4 , 7g K_2HPO_4 , 1g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1g $(\text{NH}_4)_2\text{SO}_4$, 1ml trace elements solution, 1.0ml polypropylene glycol (Merck 29767 6Y), and 20g glucose. The trace elements solution contained, per litre: 250ml conc. HCl, 3.6g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 2.0g ZnO, 0.85g $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 2.0g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 5.4g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 2.4g $\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$, 4.8g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, and 0.3g H_3BO_3 . Control was with Biolab II controllers (Brighton systems). The fermenters were grown at 25°C, pH control between 6.9 and 7.1 (with 5M NaOH/ H_3PO_4), 800 rpm, air flow at 1.0ml.min⁻¹ for 40 hours. Cell pastes were harvested by centrifugation at 9,000g, 4°C for 20 minutes and were stored at -20°C for future use.

10 **Example 3** *exo*-3-Aza-tricyclo[4.2.1.0^{2,5}]non-7-en-4-one

A solution of 20.3g norbornadiene in 100ml dichloromethane was slowly added, with stirring, to a solution of 19.1ml chlorosulfonyl isocyanate in 40ml dichloromethane. Upon addition, the solution turned red and gradually darkened to a deep purple colour. After the addition was complete, the mixture was left stirring for a further 30 minutes.

15 The solution was then slowly added to a stirred mixture of 75ml of 33% sodium sulfite solution and 50ml dichloromethane, keeping the temperature at 25°C. The reaction mixture was left stirring for a further 30 minutes after the addition was complete. The organic layer was then separated from the aqueous and dried over magnesium sulfate. Removal of solvent by rotary evaporator yielded 18g, 61%, of a white solid.

20 GC-MS : indicated m/z: 135 (M⁺), 107, 91, 70.

Example 4 Bioresolution of *exo*-3-Azatricyclo[4.2.1.0^{2,5}]non-7-en-4-one

Into a 125ml conical flask were placed 2g lactam, 40ml of 50mM phosphate buffer, pH 7 and 0.5g *P. putida* CMC103381. The reaction vessel was placed inside a heated jacket to ensure that the temperature remained at 25°C. The reaction mixture was gently stirred and, after 24 hours, a further 1.5g of *P. putida* cells were added to the reaction mixture. After a total reaction time of 39 hours, an aliquot from the reaction was found by chiral GC to contain only a single enantiomer of the cyclic β-lactam. The reaction was halted after 46 hours, the enzyme cells were spun out by centrifugation (3400 rpm) and the resulting pellet was washed with distilled water and respun. The supernatant was extracted with 2 x 70ml ethyl acetate, the organic extracts were combined and dried over magnesium sulfate. Removal of solvent by rotary evaporator yielded 770mg of a

white solid, $[\alpha]_D = -91^\circ$ (20°C, MeOH). Chiral GC analysis was carried out using the Chirasil Dex-CB column: Retention time: 4.77 minutes, enantiomeric excess >95%.

Example 5 7-Azabicyclo[4.2.0]-oct-4-en-8-one

A solution of 10ml of cyclohexa-1,3-diene in 35ml of dichloromethane was slowly added, with stirring, to a solution of 7.4ml of chlorosulfonyl isocyanate in 150ml of dichloromethane. Upon addition, the solution turned red and gradually darkened to a deep purple colour. After the addition was complete, the mixture was left stirring for a further 5 minutes. The solution was then slowly added to a stirred mixture of 100ml of 25% sodium sulfite solution and 50ml dichloromethane. The reaction mixture was left stirring for a further 20 minutes after the addition was complete. The organic layer was then separated from the aqueous and dried over magnesium sulfate. Removal of solvent by rotary evaporator yielded 3.4g, 33%, of a yellow oil which solidified on standing.

GC-MS : indicated m/z: 123 (M^+), 94, 80

1H NMR ($CDCl_3$): 6.3-5.8 (2H; m), 4.1 (1H; t), 3.5 (1H; brs), 2.3-1.3 (4H; m).

Example 6 Bioresolution of 7-azabicyclo[4.2.0]-oct-4-en-8-one

Into a 100ml conical flask were placed 1.49g lactam, 40ml of 50mM phosphate buffer, pH 7 and 1.41g *P. putida* CMC103381. The reaction vessel was placed within a heated jacket and the temperature was maintained at 30°C. The reaction mixture was stirred continuously for 22 hours, after which time an aliquot was taken and analysed by chiral GC to determine whether the resolution was complete. The aliquot was extracted with ethyl acetate and derivatised using TFAA. The resulting chiral GC chromatogram indicated lactam with an e.e. of 93%. The reaction was halted, enzyme cells were spun off by centrifuge (3400 rpm) and the supernatant was extracted with 3 x 50ml of ethyl acetate. The organic phase was dried over magnesium sulfate and solvent was removed by rotary evaporator to yield 460mg of an off-white solid, $[\alpha]_D = -105.5^\circ$ (20°C, MeOH).

Chiral GC analysis was carried out using a Chirasil Dex-CB column: Retention times: 15.46 mins (major enantiomer), 15.79 mins (minor enantiomer). Enantiomeric excess: 93%.

The amino acid produced from the bioresolution of 7-azabicyclo[4.2.0]-oct-4-en-8-one was isolated in two steps as the hydrochloride salt.

The 15ml of buffered solution remaining from the bioresolution described above contain 1g (7.1mmol) of amino acid (maximum). To the stirred buffer solution was slowly

added, at 10°C, 1.55g (7.1mmol) dibutyl dicarbonate dissolved in 20ml THF. The pH of the solution was maintained at 9 by addition of 3M NaOH solution. The reaction mixture was allowed to warm up to room temperature and left stirring overnight. The reaction was halted after 18 hours and THF was removed by rotary evaporator. The aqueous reaction mixture was acidified to pH3 with 10% potassium hydrogen sulfate solution. The mixture was then extracted with 3 x 50ml of ethyl acetate and the combined organic extracts were dried over magnesium sulfate. Removal of solvent by rotary evaporator yielded 1.1g, 62% of a white solid.

Into a 25ml conical flask was placed 0.71g (2.81mmol) of Boc-protected amino acid, dissolved in 15ml THF. The stirred reaction mixture was acidified to pH 3 with 3M HCl solution and the reaction was vigorously stirred for 3 hours at ambient temperature. The reaction mixture was left to stand overnight and extracted with 10ml ethyl acetate. The aqueous phase was concentrated under vacuum to yield 480mg, 87% yield of an off-white solid.

¹H NMR (CD₃OD): 6.05-5.85 (1H; m), 5.65-5.50 (1H; m), 3.9 (1H; brs), 2.85 (1H; dt), 2.15-1.65 (4H; m).

Example 7 6-Azabicyclo[3.2.0]hept-3-en-7-one

Cyclopentadiene was freshly prepared by thermolysis of cyclopentadiene dimer. It was stored at -20°C, until required to prevent dimerisation. A solution of cyclopentadiene (1.65g, 25mmol) in dichloromethane (15ml) was slowly added to a stirred solution of chlorosulphonyl isocyanate (1.8ml, 21mmol) in dichloromethane (35ml) at -20°C for 15 minutes. Upon the addition the solution turned to dark red. The temperature was kept -20°C. After a further 20 minutes, a solution of anhydrous sodium bisulfite (6g in 25ml water) was added dropwise to the stirred solution. After 30 minutes the organic layer was separated and the aqueous layer was extracted twice with dichloromethane (15ml). The combined organic phases were dried over magnesium sulfate then evaporated. ¹H-NMR showed a 55/1 mixture of the 6-azabicyclo[3.2.0]hept-3-en-7-one and the 2-azabicyclo[2.2.1]hept-5-en-3-one. The products were separated by chromatography on silica gel. 6-Azabicyclo[3.2.0]hept-3-en-7-one was isolated as a yellow oil (0.95g, 40%).

Example 8 Bioresolution of racemic 6-azabicyclo[3.2.0]hept-3-en-7-one

In a jacketed vessel at 30°C were placed 10ml phosphate buffer (50 mM, pH 7), 200mg 6-azabicyclo[3.2.0]hept-3-en-7-one and *P. putida* CMC103381 (200mg). The

reaction was stirred overnight. The ee of the residual substrate was assayed by chiral gas chromatography using a TFAA-derivatised sample. An aliquot was taken after 22h, derivatised using TFAA and analysed by GC.

Chiral Gas Chromatography, column Chirasil Dex CB

- 5 Racemate : enantiomer 1 retention = 4.09 min, enantiomer 2 t = 4.40 min.

Sample of the bioresolution reaction : GC chromatogram revealed almost exclusively single enantiomer (enantiomer 1 t = 4.09 min), giving residual substrate of >98%ee.

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